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The Ability of *Salmonella typhimurium* to Produce the Siderophore Enterobactin Is Not a Virulence Factor in Mouse Typhoid

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One of the nonspecific defense mechanisms of higher animals is their ability to limit iron availability to infecting bacteria. Thus it has been argued that all pathogenic bacteria must have special mechanisms to obtain iron in the host environment. *Salmonella typhimurium* is known to produce a siderophore, enterobactin, with which it can obtain iron from host transferrin. Previous studies have indicated that the production of this molecule is necessary for the ability of intraperitoneally injected *S. typhimurium* cells to cause mouse typhoid, a largely intracellular infection. We have reexamined this finding with wild-type *S. typhimurium* and isogenic strains carrying the nonenterobactin-producing mutation *ent-1* or *ent-7*. Our findings demonstrate that, although enterobactin production is necessary for growth in normal mouse serum, it does not affect the ability of *S. typhimurium* to cause mouse typhoid. Based on these findings and published results of other investigators on the role of siderphores in intracellular pathogens, a more comprehensive investigation of the importance of siderophores in intracellular infections may be warranted.

Siderophores are small, diffusible molecules produced by many bacteria which allow them to acquire iron from their environment (30, 31). Although there have been some reports to the contrary (36, 40), it is widely believed that the ability of a pathogen to use siderophores to extract iron from a host is necessary in virtually all pathogenic infections (10, 44). In the case of *Salmonella typhimurium*, one of the best-documented studies of the importance of siderophores for virulence has been a study showing that a strain of *S. typhimurium* that had lost its ability to make the iron-binding siderophore enterobactin (*ent*) failed to cause mouse typhoid (47), a disease known to be largely intracellular (27, 28).

Resistance to infection based on the ability of a pathogen to obtain nutrients from the host was formulated by Garber as the "nutrition-inhibition hypothesis of pathogenicity" (12). Later, Kochan termed the specific cases where a host limits the availability of required microbial nutrients "nutritional immunity" (21). The competition between vertebrate hosts and microbes for iron, which is required by all pathogens examined, is the best-studied example of nutritional immunity (3, 9, 10, 43). Mammals, birds, and reptiles all maintain very low free iron in body fluids by binding iron with high-affinity proteins such as transferrin in serum, lactoferrin in milk and other secretions, and conalbumin in egg white (14, 43). These proteins are all structurally related (2) and have a high affinity for Fe^{3+}, resulting in levels of available free iron (about 10^{-18} M) that are much too low for growth of most microbes.

Invasive pathogens produce iron-harvesting siderophores that allow them to compete with these host proteins for iron. In an effort to limit iron acquisition by siderophore-producing bacteria, mammals respond to very small amounts of lipopolysaccharide or other inflammatory agents by sequestering much of the normally available serum iron as ferric ferritin in reticuloendothelial macrophages, primarily in the spleen and liver (11, 21). These same inflammatory agents also cause fever in most vertebrates (19), which decreases the ability of several bacterial pathogens, including *S. typhimurium*, to produce iron-harvesting siderophores (13, 20, 22, 46) and to take up ferric enterobactin (46).

Virulence of a large number of pathogens is increased if iron is given at the time of infection (3, 9, 10, 44). This has been used as evidence that low iron is a limiting factor in growth of the pathogen. In addition, the ability to produce siderophores has been reported to be important for the virulence of *Escherichia coli* (45), *S. typhimurium* (47), and *Vibrio anguillarum* (8). There is some evidence to suggest that the production of siderophores is also important for the virulence of *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* (33, 48). *Neisseria meningitidis* apparently does not produce sufficient siderophores to support growth in serum, but has a specific receptor for transferrin on its surface which allows it to acquire enough iron from transferrin for growth in normal serum (9, 38). In the case of the intracellular parasites *Legionella* spp. (36) and *Yersinia* spp. (40), there is evidence suggesting that high-affinity iron-harvesting systems are not needed after the pathogens are in macrophages.

In this study we reexamined the importance of the enterobactin siderophore produced by *S. typhimurium* (30) in the pathogenesis of mouse typhoid. We produced isogenic *S. typhimurium* strains which were *ent* and did not have the ability to produce enterobactin or other high-affinity siderophores. The *ent* mutations, *ent-1* and *ent-7*, were separately transferred into the mouse-virulent *S. typhimurium* SL1344. We observed that even though the enterobactin-negative exconjugants grew very poorly in normal mouse serum and in a medium where the iron was chelated by citrate, they grew as well as their enterobactin-producing parent in several inbred mouse strains. Based on these results, we concluded that siderophores were not important in the pathogenesis of mouse typhoid, and that they may not be important in other intracellular infections.

MATERIALS AND METHODS

Mice. All mice were obtained from Jackson Laboratories, Bar Harbor, Maine, and were used at 8 to 12 weeks of age. Mice were maintained for at least 2 weeks on Prolab

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TABLE 1. *S. typhimurium* strains

Designation	Relevant genotype	Source
SL1344	*hisG46*	Hoiseth and Stocker (16)
SL3261	*hisG46 aroA*	Hoiseth and Stocker (16)
JL3521	*gyrA*	L. Bussey (5)
LT2-Z	Prototroph	B. Ames
WB1	*hisG46 aroA gyrA*	P22(JL3521)×SL3261[a]
Enb-1	*ent-1*	J. Neilands (34)
Enb-7	*ent-7*	J. Neilands (34)
TT289	*purE884*::Tn*10*	J. Roth (37)
WB2	*ent-1 purE884*::Tn*10*	P22(TT289)×Enb-1
WB3	*ent-7 purE884*::Tn*10*	P22(TT289)×Enb-7
TT628	*pyrC7*/F'(Ts)114 *lac*$^+$ *zzf-22*::Tn*10*B	J. Roth (7)
WB4	*ent-1 purE884*::Tn*10*/ F'(Ts)114 *lac*$^+$ *zzf-21*::Tn*10*B	TT628×WB2[b]
WB5	*ent-7 purE884*::Tn*10*/ F'(Ts)114 *lac*$^+$ *zzf-21*::Tn*10*B	TT628×WB3
WB6	*ent-1 hisG46 gyrA*	WB4×WB1
WB7	*ent-7 hisG46 gyrA*	WB5×WB1
WB9	*hisG46 gyrA*	P22(LT2-Z)×WB7
WB10	*hisG46 gyrA*	P22(LT2-Z)×WB7

[a] Designation indicates transductional crosses; for example, P22(JL3521)×SL3261 indicates that SL3261 was infected with a P22 lysate grown on JL3521 and selected for the phenotype of interest, in this case nalidixic acid resistance (*gyrA*).

[b] Designation indicates conjugational mating; for example, donor strain TT628 was mated with WB2. Selection in this case was for Pyr$^+$ and Lac$^+$, which yields an exconjugant in which F'(Ts)114 *lac*$^+$ *zzf-21*::Tn*10*B was transferred from TT628 to WB2.

RMH1000 (Agway Inc., Syracuse, N.Y.) mouse pellets ad libitum in plastic cages with stainless steel tops. Several of the mouse strains were F_1 hybrids. They were CAF_1, $(BALB/cJ \times A/J)F_1$; $B6D2F_1$, $(C57BL/6J \times DBA/2J)F_1$; $CB6F_1$, $(BALB/cJ \times C57BL/6J)F_1$; and LAF_1, $(C57L/J \times A/J)F_1$.

Bacteria. The *S. typhimurium* strains used in this study are listed in Table 1. SL1344 was derived from a calf isolate. SL3261 is an *aroA* derivative of SL1344 which was produced by selecting for a transposon Tn*10*-mediated deletion in *aroA* (16). All others were derived from LT2. Hfr (high frequency mating) strains WB4 and WB5 were prepared as described by Chumley et al. (7).

Media. Nutrient broth (NB) contained 8 g of nutrient broth (BBL Microbiology Systems, Cockeysville, Md.) and 5 g of NaCl per liter. The E medium of Vogel and Bonner supplemented with 0.2% glucose was used as a high-citrate (10 mM) minimal medium to inhibit growth of *ent* strains (41). Media used for selection of transductants and exconjugants in the strain constructions described in Table 1 were NCE medium, which was a modification of the E medium without citrate (7) and was supplemented with 0.2% lactose to select for growth on this sugar (lactose) as the sole carbon source. For solid media, 1.5% BBL agar was added. Antibiotics were added to nutrient agar at 25 μg/ml for tetracycline (Sigma Chemical Co., St. Louis, Mo.) or 40 μg/ml for nalidixic acid (Sigma). The following nutrients were added to minimal medium where appropriate: L-histidine (Aldrich Chemical Co., Inc., Milwaukee, Wis.), 0.1 mM; ferric ammonium citrate (green) (Fisher Scientific Co., Pittsburgh, Pa.), 167 mg/liter (equivalent to 25 mg of Fe per liter); and 2,3-dihydroxybenzoic acid (2,3-DHB) (Aldrich), 10 mg/liter.

Transductional methods. Phage P22 HT105/1 *int201*, which is a high-frequency generalized transducing bacteriophage, was used for all transductional crosses (7). To select tetracycline-resistant transductants, nutrient agar plates containing tetracycline were spread with 10^8 bacterial cells and 10^8 to 10^9 PFU of phage P22. To transduce the *gyrA* mutation, 10^8 cells and 10^8 PFU of phage were spread on a nutrient agar plate. After 12 h at 37°C, transductants were replica plated to nalidixic acid plates. After 24 h at 37°C, colonies were picked and streaked on nalidixic acid plates to obtain isolated nalidixic acid-resistant transductants.

Conjugational methods. Hfr donor and recipient strains were grown in NB for 4 h at 37°C with no shaking and then mixed 1:2 (vol/vol). A 0.3-ml sample was plated on appropriately supplemented minimal medium containing nalidixic acid to interrupt the mating at specified times. The *ent* strains WB6 and WB7 were obtained from matings interrupted at 30 min with nalidixic acid so that no more than 22% of the chromosome was transferred. Since the Hfr strains initiate transfer of the chromosome at *purE* or 12 units of the *S. typhimurium* map and transfer it in a clockwise direction toward the selected marker *aroA*, the only part of the chromosome transferred was from 12 to about 34 units (37).

Mouse infections. *S. typhimurium* strains for infecting mice were stored at −70°C in NB with 10% dimethyl sulfoxide (Fisher). A portion of an overnight NB culture was diluted to a density of about 10^6 CFU/ml in 5 ml of fresh NB and grown for approximately 2.5 h at 37°C with shaking. Bacteria were harvested by centrifugation (at 4°C), washed once with lactated Ringer solution (Abbott), and suspended in 5 ml of Ringer solution, and the absorbance of the suspension was measured at 420 nm (an optical density of 1 equals 3×10^8 CFU/ml) with a Beckman model 25 spectrophotometer. Mice were injected in the lateral tail vein or intraperitoneally (i.p.) as indicated with 0.2 ml of lactated Ringer solution containing the indicated doses of bacteria. Bacterial concentrations were always confirmed by plating. To determine the amount of growth of the *S. typhimurium* in mice, the spleen and liver of each mouse were homogenized in 10 ml of 10 mM phosphate buffer (pH 7.2) at 4°C with a stomacher (Techmar, Cincinnati, Ohio). Samples of the homogenate were serially diluted in phosphate buffer, and 0.7-ml samples were mixed with 8 ml of liquid (50°C) bismuth sulfite agar (Difco Laboratories, Detroit, Mich.) and quickly poured into a petri dish. Plates were incubated at 37°C, and colonies were counted after 18 to 24 h. Data were expressed as the total CFU recovered per mouse. Mice allowed to die after infection were checked daily until death.

Growth of *ent*$^+$ and *ent S. typhimurium* in NMS. Normal inbred mice were bled from the orbital sinus, and the blood was immediately put on ice. The serum was harvested, filtered (0.45 μm), and frozen at −20°C within 4 h after collection. Samples (1 ml) of the serum were inoculated with 6×10^4 CFU of an overnight NB culture which had been washed as described above in Ringer solution. Samples were taken at the times indicated, diluted, and plated in bismuth sulfite as above. In some cases the normal mouse serum (NMS) was supplemented with 6 μg of iron per ml as ferric ammonium citrate or 5 μg of 2,3-DHB per ml. In neither case did supplementation increase the volume more than 1%. The percent transferrin saturation was calculated from total iron content of the serum divided by total iron-binding capacity of the particular serum pool. Total iron and total iron-binding capacity of the serum were determined on an automatic clinical analyzer III (Du Pont Co., Wilmington, Del.), which utilizes bathophenanthroline sulfonate to react with Fe^{2+} under acid conditions to form a colored reaction product which is measured at 535 nm (15).

Growth of *ent*$^+$ and *ent S. typhimurium* in the peritoneal cavity. The *S. typhimurium* cells were incubated in the indicated medium, washed, and diluted in Ringer solution as described above, and 10^4 CFU in 0.2 ml was injected i.p. in BALB/cByJ mice. At intervals, mice were killed by cervical dislocation and 3 ml of cold 4°C Ringer solution was injected i.p. The peritoneum was massaged for 1 min, a 0.1-ml portion of the fluid was removed and diluted, and the number of viable *S. typhimurium* was determined as described above.

RESULTS

Growth of mouse-virulent *S. typhimurium* SL1344 and isogenic *ent* strains in NMS. The *ent* strains WB6 and WB7 were constructed by conjugation as described in Table 1. Exconjugants (Aro$^+$) were screened for a requirement for either iron or 2,3-DHB for growth on medium E; approximately 50% of the exconjugants in both constructions displayed this requirement. Strains WB6 and WB7 could be cross-fed by an enterobactin-producing strain on a medium E plate. Consistent with the parental Hfr origin of the *ent* mutations, strain WB6 required added iron (class I *ent* mutant) and WB7 required either iron or 2,3-DHB (class II *ent* mutant) for growth on citrate-containing medium E. The growth of these two strains was no better on the E medium than was growth of the parental strains Enb-1 and Enb-7 (data not shown). These experiments demonstrate that the exconjugants do not produce any high-affinity siderophores that could compete with citrate for iron.

Figure 1 shows the growth of the *ent S. typhimurium* strains in normal mouse serum; both of the *ent* strains grew much less than the *ent*$^+$ parent strain. The *ent* strains grew at about the same rate as the wild type for the first 6 h. This growth apparently exhausted the intracellular iron; without supplementation, growth ceased after about four generations. Essentially the same results were obtained with three different pools of NMS. The pool used in these experiments had 67% of the iron-binding capacity saturated. WB7 grew when supplemented with either 2,3-DHB or iron, whereas

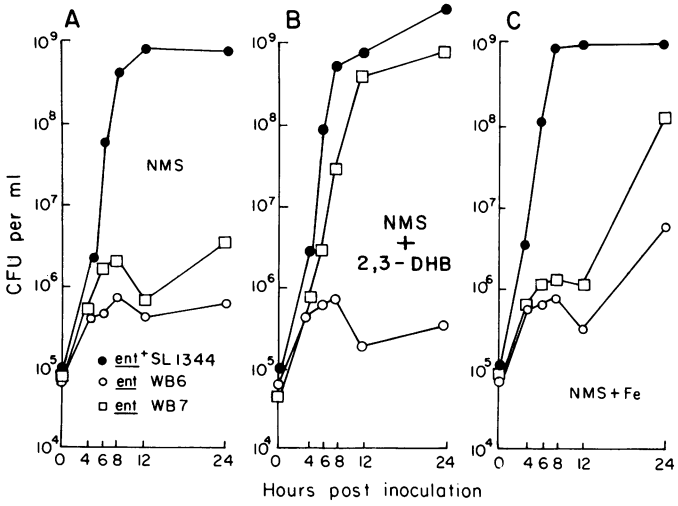

FIG. 1. Relative growth of *ent* strains WB6 and WB7 and their parent *ent*$^+$ strain SL1344 in 1 ml of (A) NMS, (B) NMS supplemented with 5 μg of 2,3-DHB per ml, or (C) NMS supplemented with 6 μg of Fe per ml. Cultures were inoculated at time zero, and 50-μl samples were removed at 4, 6, 8, 12, and 24 h.

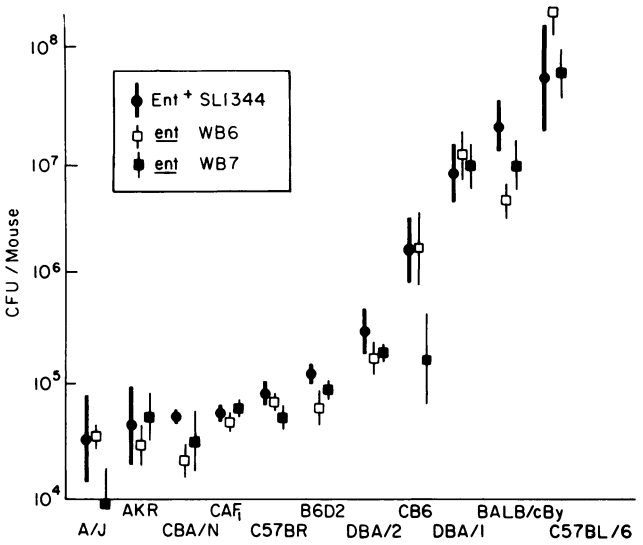

FIG. 2. Net growth of *ent* strains WB6 and WB7 and their *ent*$^+$ parent in inbred and F_1 hybrid mouse strains. Mice were infected i.v. with 100 CFU of *S. typhimurium*. The total numbers of *S. typhimurium* in their spleens and livers were determined 5 days later. Means of 4 to 17 mice ± standard errors are shown.

WB6 grew only in serum supplemented with iron. Interestingly, both *ent* strains showed a lag in growth when placed in serum supplemented with iron. Although the reason for this is not clear, it may have occurred when the intracellular stores of iron were depleted and lasted until some unknown metabolic readjustment occurred that allowed growth in the new environment. Whatever change occurred, it was not a reversion of the enterobactin mutation, because no enterobactin-positive isolates were recovered from the cultures at the completion of the experiment.

Growth of i.v.-inoculated *ent S. typhimurium* in inbred mice. Mice from 11 inbred mouse strains were infected intravenously (i.v.) with the *ent S. typhimurium* strains WB6 and WB7 to compare their growth with the growth of their mouse-virulent parent SL1344. The *ent* strains in this study generally grew as well as or better than the virulent parent SL1344 in mice (Fig. 2). One isolated variation from this pattern was observed, however. WB7 grew significantly less in $CB6F_1$ mice. Since the other *ent* strains, WB6, grew as well as the *ent*$^+$ parent, SL1344, it seems likely that the decreased growth of the WB7 was not associated with the enterobactin-negative phenotype. Four colonies from each of over 40 mice infected with WB6 and WB7 *S. typhimurium* were checked for their *ent* phenotype, and in no case did we find either an *ent*$^+$ or the class of *ent* mutation other than the one the mouse was infected with.

Days to death of mice infected i.v. with *ent* and *ent*$^+$ *S. typhimurium*. To investigate the possibility that an observable effect of the *ent* phenotype on virulence might be more readily seen at high inocula, we examined the survival of LAF_1 mice infected with *ent*$^+$ and *ent S. typhimurium*. These mice were chosen since they were relatively resistant to *S. typhimurium* infection and would survive a high load of *S. typhimurium* longer than more susceptible mouse strains. We hoped that this property of LAF_1 mice would maximize their opportunity to regulate their iron levels before becoming moribund. There was no significant difference in the days to death of mice infected with *ent* or *ent*$^+$ *S. typhimurium* strains at any of the doses used (Fig. 3).

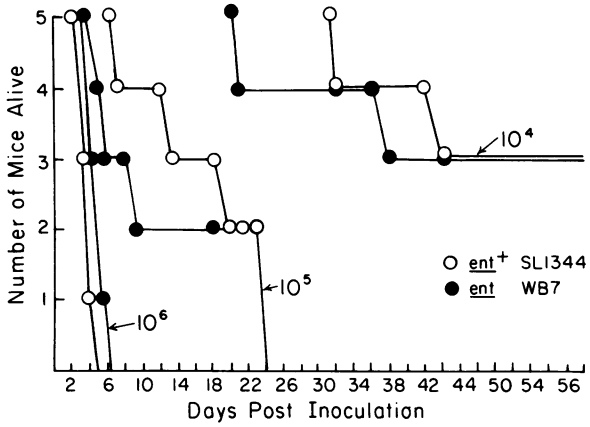

FIG. 3. Mortality of LAF_1 mice infected with the indicated numbers of *ent*$^+$ (SL1344) and *ent* (WB7) *S. typhimurium* organisms.

Growth of iron-starved *ent S. typhimurium* in mice after i.v. inoculation. To examine the possibility that *ent S. typhimurium* grown in nutrient broth may store sufficient iron to permit them to kill mice without requiring more iron in vivo, we infected mice with iron-starved bacteria. To obtain iron-starved *ent S. typhimurium*, we inoculated E medium without added iron or 2,3-DHB from an overnight NB culture of WB7. This culture was incubated for 22 h at 37°C. Cell growth ceased in less than three generations. As a control, we prepared histidine-starved WB7. E medium supplemented with 2,3-DHB, but without added histidine, was inoculated from the same overnight NB culture of WB7. After 22 h of incubation, the culture contained only 86% as many CFU as there were in the initial inoculum. A third E medium culture of WB7 was supplemented with excess histidine and 2,3-DHB. This medium supported 20 generations of growth in 22 h. Three groups of five BALB/c mice were each infected with 900 CFU of one of these bacterial preparations, and the number of bacteria in the surviving mice was determined on day 6. There was extensive in vivo growth of all three WB7 preparations in mice, with no significant difference between the results obtained with the histidine- or iron-starved preparations and the nonstarved culture (Table 2). This result indicated that the *S. typhimurium* cells were able to acquire iron (and histidine) in vivo in spite of the *ent* mutation.

TABLE 2. In vivo growth of iron-starved *ent S. typhimurium* WB7

E medium supplemented with:	Nutritional state of *S. typhimurium*	Net generation in medium[a]	CFU recovered at 6 days postinoculation[b]
Histidine + 2,3-DHB	Normal	20.9	9.9×10^7, 1.6×10^8, dead, dead, dead
2,3-DHB	Starved for histidine	<1[c]	3.2×10^7, 6.5×10^8, 7.2×10^8, dead, dead
Histidine	Starved for iron	2.4	2.0×10^5, 7.1×10^7, 8.1×10^7, 8.4×10^7, dead

[a] Generations in vitro during the 22 h before bacteria from these cultures were used to inoculate mice.
[b] *S. typhimurium* recovered 6 days after mice were given 900 CFU i.v.
[c] Fourteen percent less CFU were recovered than were in the original inoculum.

TABLE 3. Mortality of mice infected i.p. with *ent*$^+$ and *ent-7 S. typhimurium*[a]

Bacterial strain	Enterobactin production	Median day of death for i.p. inoculation dose[b]:	
		10^2 CFU	10^3 CFU
SL1344	+	6.6	5.2
WB7	−	6.9	5.6
WB9	+	6.4	6.2
WB10	+	6.9	6.1

[a] Five BALB/cJ mice for each data point were inoculated i.p. with 10^2 or 10^3 CFU of the indicated *S. typhimurium* strain. All mice died between days 5 and 8 after inoculation.
[b] Median day of death was determined by calculating the reciprocal mean day of death for the five mice in each group. The two-sample rank test was used to compare the time of death in each group. None showed a time of death significantly different from that of mice inoculated with *ent S. typhimurium* WB7.

The foregoing studies showing a lack of effect of *ent* on the virulence of *S. typhimurium* in mice are in contrast to the results of Yancy et al. (47). We felt that one explanation for this might be that Yancey's inoculations were i.p. rather than i.v. *S. typhimurium* cells injected i.p. might be expected to remain extracellular for a longer period of time than those injected i.v. Since extracellular *S. typhimurium* would be exposed to an environment similar to NMS, the *ent*$^+$ strains would be expected to show more rapid growth than the *ent* strains (Fig. 1).

Virulence of *ent S. typhimurium* after i.p. inoculation. To determine whether the inability to make siderophores would decrease virulence after i.p. injection, we infected BALB/By mice with 10^2 or 10^3 CFU of *ent* WB7, *ent*$^+$ SL1344, and two *ent*$^+$ transductants of WB7, WB9 and WB10, and recorded the day of death. All mice died by day 8 after i.p. infection with either 10^2 or 10^3 CFU. There was no significant difference in the time to death of mice infected with *ent* strain WB7 as compared with those infected with its *ent*$^+$ isogenic partners (Table 3).

We also followed the growth of *ent* and *ent*$^+$ strains for up to 12 h after i.p. inoculation in the peritoneal cavity. When the inoculum was grown in NB, *ent*$^+$ WB9 grew slightly more than *ent* WB7 (Table 4). We thought that this slight difference may be enhanced if the *ent* WB7 strain were starved for iron. WB7 was cultured in E medium without 2,3-DHB, as described above, and inoculated i.p. As controls, WB7 and *ent*$^+$ WB9 were grown in the absence of histidine. When the mice were killed at 8 and 12 h postinoculation, slightly lower numbers of CFU were recovered from the mice infected with iron-starved bacteria versus those receiving the histidine-starved *S. typhimurium*. In the case of the comparison between WB7 grown without 2,3-DHB or without histidine, the difference was significant, although only twofold. Taken together, the data in Tables 3 and 4 indicate that, although *ent* may play a small role in the growth of i.p.-inoculated *S. typhimurium*, it has no effect on the eventual mortality of the inoculated mice.

DISCUSSION

The results of this study show that the production of an iron-binding siderophore is not required for full virulence of mouse-virulent *S. typhimurium* given parenterally. These results were surprising in light of the well-known importance of iron in infectious processes (3, 9, 10, 43). They were also at variance with a report of Yancy et al. (47), who found reduced virulence in outbred mice of an *ent* mutant of *S.*

TABLE 4. Growth of *ent*$^+$ and *ent S. typhimurium* in the peritoneal cavity[a]

Expt[b]	*S. typhimurium* strain	Growth medium	No. of CFU recovered from the peritoneal cavity relative to total injected[c]: 4 h	8 h	12 h
1	WB7	NB	3.3 (1.3) 13	3.1 (1.5) 12[d]	
	WB9	NB	3.0 (1.6) 13	5.1 (1.5) 13	
2	WB7	E − 2,3-DHB		1.3 (2.6) 2	1.8 (1.1) 5[d]
	WB7	E − histidine		4.0 (1.3) 2	3.8 (1.2) 5
	WB9	E − histidine		2.3 (1.6) 2	2.2 (2.2) 6

[a] WB7 (*ent*) or WB9 (*ent*$^+$) *S. typhimurium* (10^4 cells) were injected i.p. into BALB/cByJ mice. Mice were killed either 4, 8, or 12 h postinoculation, and from the number of CFU in a peritoneal wash the total free CFU in the cavity was determined.

[b] In experiment 1 the *S. typhimurium* cells were from an overnight NB culture. In experiment 2 the medium was the E medium of Vogel not supplemented with one nutrient required for growth of the *S. typhimurium*; thus all strains had stopped growing because of a lack of histidine or 2,3-DHB, a precursor of enterobactin that WB7 can utilize. The deficient nutrient is indicated.

[c] Data are expressed as the geometric mean CFU recovered divided by the CFU injected. The numbers within parenthesis are the standard error factors and are the numbers by which the geometric mean must be multiplied and divided to obtain the upper and lower standard error bounds. The third number indicates the number of samples.

[d] The data for WB7 versus WB9 (experiment 1) or WB7 minus 2,3-DHB versus WB9 minus histidine (experiment 2) were not significantly different by the *t* test ($P > 0.05$). At 12 h the difference between WB7 minus histidine versus WB7 minus 2,3-DHB was significant ($P < 0.05$) by the *t* test.

typhimurium (SR-11 96.1) that was derived by mutagenesis. Although we were unable to test their finding with an identical group of outbred mice, we have tested several inbred mouse strains and outbred mice from three vendors, including the one used by Yancey et al., and never found reduced virulence of their *ent* strain SR-11 96.1 as compared with its parent, SR-11 (data not shown). In fact, we found that SR-11 96.1 did not lend itself to in vivo studies since the *ent-96.1* mutation was leaky as compared with the *ent-7* mutation, a finding Yancey (Moore et al.) has already reported (29).

Within 2 h of i.v. inoculation, over 98% of the injected *S. typhimurium* organisms had been cleared from the blood, and virtually all of the surviving *S. typhimurium* organisms were associated with cells of the spleen and liver (39; Benjamin et al., submitted for publication). Thus, it was likely that the failure of the *ent* mutation to affect the virulence of *S. typhimurium* in mice was because it had no effect on the growth rate of *S. typhimurium* cells once they had entered cells of the reticuloendothelial system.

This conclusion was in agreement with that of others who observed that intracellular pathogens do not need high-affinity iron-gathering processes for virulence (4, 26, 36, 40). For example, *Yersinia* spp. are greatly attenuated when they are deficient in one of two iron-gathering systems when injected i.p. without added iron, but are fully virulent when they are given i.v. and are thus taken up rapidly enough by the cells of the reticuloendothelium to minimize their extracellular growth phase (40). The data presented here show that *S. typhimurium* strains which were unable to produce any siderophores, and were thus unable to grow in the presence of the weak iron chelator citrate, were as fully virulent as their *ent*$^+$ isogenic partners whether given i.v. or i.p. This suggests that the extracellular phase of growth is less important for *S. typhimurium* than for *Yersinia* spp. *Legionella* species, which also grow in macrophages, have been found not to have any high-affinity iron-gathering systems, which may even limit their growth in the relatively iron-rich intracellular environment (36).

At first glance, the hypothesis that high-affinity siderophores do not play an important role in the pathogenesis of *S. typhimurium* for mice is contrary to a large body of data indicating that alterations in serum iron levels affect the pathogenesis of a large number of bacteria, including *S. typhimurium* (1, 3, 10, 17, 25, 32, 35, 44). Iron deficiency, either dietary or chelator induced, has been shown to decrease the severity of diseases caused by *S. typhimurium* (17, 35, 44). When serum iron levels are elevated either experimentally or due to hemolytic disease the severity of bacterial infections, including those caused by *S. typhimurium*, is universally increased (1, 3, 7, 10, 25, 32, 44). We feel that this large body of data does indicate an effect of iron levels on pathogenesis, but that, at least in the case of *S. typhimurium*, the different serum iron levels probably exert their effect either by modulating the immune system (18, 23) or by changing the levels of iron intracellularly, where siderophores are not required for its acquisition.

In only two experimental systems, other than the *S. typhimurium* model discussed above, have siderophores been shown to be important for virulence. In both cases the infections caused by these pathogens are extracellular. Neither of the siderophores in these systems is enterobactin, and, although they are encoded on a plasmid in each case, the siderophores are apparently different (8, 42, 45). One is the ColV plasmid, which codes for the siderophore aerobactin and is usually found in enterobactin-producing strains of *E. coli* (45), causing increased virulence. The other siderophore is coded for by the plasmid pJM1, which is necessary for virulence of the fish pathogen *Vibrio anguillarum*. The siderophore, as yet unidentified, is the only siderophore produced by the pathogen (8, 42). Thus, no convincing data exist indicating that the siderophore enterobactin is important for intracellular or even intravascular infection. In fact, a report by Konopka and Nielands indicates that enterobactin is much less effective than aerobactin at transferring serum iron to *E. coli* (24). Perhaps the role of enterobactin produced by *Enterobacteriaceae* is to aid commensals while growing in the gut and in other environments, rather than in the blood and tissues of higher organisms (31).

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